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REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	2/19/97	Final

4. TITLE AND SUBTITLE
Studies on Bacterial Spore Resistance and Regulation of Activation

5. FUNDING NUMBERS
DAAH04-93-G-0263

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8. PERFORMING ORGANIZATION REPORT NUMBER

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U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
ARO 31522.13-LS

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12 b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*
Highlights of work on spores of Bacillus subtilis in the past performance period have included demonstration that: 1) the zymogen of the spore protease GPR autoactivates, and that autoactivation is stimulated by low pH, dipicolinic acid and dehydration; 2) most of the propeptide of GPR is not needed for zymogen stability or autoprocessing; 3) the decrease in forespore pH in B. subtilis sporulation is responsible for 3-phosphoglycerate accumulation; 4) α/β-type small, acid-soluble proteins in spores protect DNA against killing by freeze-drying, dry heat and several organic hydroperoxides, by preventing DNA damage; 5) DNA repair during spore germination is an important component of resistance of spores to agents that kill spores through DNA damage; and 6) spore resistance to peroxides is not due to the presence of protective enzymes such as catalases or alkylhydroperoxide reductases in spores.

14. SUBJECT TERMS	15. NUMBER IF PAGES
Spores; DNA repair; spore resistance; protease	
	16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

REPORT DOCUMENTATION PAGE (SF298)
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1. Problem Studied

There were two major problems to be studied in the past performance period: 1) the mechanism of bacterial spore resistance; and 2) the mechanism of regulation of a spore specific protease (termed GPR) which acts in the first minutes of spore germination on α/β-type small, acid-soluble spore proteins (SASP), GPR is initially made as a zymogen.

a) Spore resistance -

Major specific aims in this area were TO: 1) determine the relationship between effects of various killing agents on the viability, loss of dipicolinic acid, loss of ability to germinate and amount and type of DNA damage in wild type spores of Bacillus subtilis as well as in mutant spores (termed $\alpha^-\beta^-$) lacking the majority of their DNA protective α/β-type SASP; 2) analyze mutants generated by heat and hydrogen peroxide in $\alpha^-\beta^-$ spores to determine the sequence changes generated by these agents, and thus gain insight into the DNA damage caused by these treatments; 3) determine the importance of DNA repair in resistance to various treatments of both wild-type and $\alpha^-\beta^-$ spores; 4) determine the role (if any) of protective enzymes such as catalases in spore resistance; and 5) continue to analyze the structure of the α/β-type SASP-DNA complex.

b) GPR

Major specific aims in this area were to: 1) analyze GPR mutants lacking the propeptide for their activity in vivo during sporulation; 2) examine various forms of GPR for any C-terminal processing; 3) determine the active site residue(s); 4) engineer the site at which the GPR zymogen (termed P_{46}) is cleaved to the active enzyme (termed P_{41}) to make this sequence more like that cleaved by GPR in α/β-type SASP; 5) generate large amounts of purified P_{46} and P_{41} for physical studies; 6) elucidate conditions to obtain processing of P_{46} to P_{41} in vitro; and 7) identify the enzyme that removes the amino-terminal leucine residue from P_{41}.

As is noted in 2 below, we have made great progress on many of these aims, and have also initiated several new areas of research.

2. Summary of Important Results

Important results obtained during the past performance period include the following: a) α/β-Type SASP were shown to protect spores from DNA damage due to freeze drying. b) Wild-type spores were shown to accumulate DNA damage, probably DNA depurination, upon dry heat treatment. However, $\alpha^-\beta^-$ spores were killed at much lower temperatures than were wild-type spores, and α/β-type SASP protected DNA in vitro against dry heat induced DNA depurination. c) Killing of wild-type spores by organic hydroperoxides was not by DNA damage. However, $\alpha^-\beta^-$ spores were more sensitive to two organic hydroperoxides (cumene and t-butylhydroperoxide) and were killed by DNA damage. In contrast, $\alpha^-\beta^-$ and wild-type spores exhibited identical sensitivity to peracetic acid, and were not killed by DNA damage. d) Treatments of spores that resulted in killing by DNA damage resulted in induction of expression of DNA repair genes such as uvrB, dinR, and recA during subsequent germination and outgrowth of the treated spores. In contrast, treatments that did not kill spores by DNA damage did not result in induction of these DNA repair genes. e) A mutation in the recA gene whose product is responsible for turning on many other DNA repair genes generally reduces spore resistance to treatments that kill by DNA damage, but not treatments that kill by other mechanisms. Thus killing of wild-type spores by wet heat, hydrogen peroxide, or organic hydroperoxides is not affected by a recA mutation nor is killing of $\alpha^-\beta^-$ spores by peracetic acid. In contrast a recA mutation greatly increases the sensitivity of wild-type spores to dry heat, and the sensitivity of $\alpha^-\beta^-$ spores to wet and dry heat, freeze-drying, hydrogen peroxide, cumene hydroperoxide and t-butylhydroperoxide. f) Elimination of a number of enzymes that could potentially protect spores against oxidative stress had no effect on spore resistance. Enzymes that have been tested to date include catalases A and B, both alkylhydroperoxide reductases, and superoxide dismutase. g) Mutants in the gpr gene lacking the prosequence make an enzyme that is fully active in vivo and in vitro. However, mutants retaining only five of the 15 propeptide residues make an enzyme that is still a

zymogen, but this zymogen processes normally to the active enzyme. h) Mutagenesis of the gpr gene such that the $P_{46} \rightarrow P_{41}$ cleavage site is more like that cleaved by GPR in SASP results in an enzyme that spontaneously processes to P_{41} when the alteered genes are expressed in either B. subtilis or E. coli. The latter result strongly indicates that P_{46} processes itself to P_{41}. i) Studies in vitro with purified P_{46} showed that the zymogen can autoprocess correctly to P_{41} in an intramolecular reaction. Although the zymogen is indefinitely stable under normal physiological conditions, autoprocessing is stimulated by a decrease in pH to 6-6.5, a high level of dipicolinic acid (and only the biologically important 2, 6 isomer), and dehydration. All of these conditions are those in the developing forespore at the time of $P_{46} \rightarrow P_{41}$ conversion in vivo. j) Large amounts of highly purified P_{46} have been provided for crystallization. Crystals have been obtained, but in preliminary work they do not diffract. k) More detailed analysis of the response of purified GPR to inhibitors has shown that the enzyme is likely not a serine protease. In mutagenesis experiments to date the enzyme's single SH group has been shown not to be essential for catalysis. l) Studies of the pH within the developing sporangium of B. subtilis showed that the forespore pH falls to ~ 6.5 ~ 1 hr before dipicolinic acid accumulation, and just before 3-phosphoglycerate (3PGA) accumulation. All sporulation mutants which failed to show the pH decrease failed to accumulate 3PGA, while mutants that did show the pH decrease also accumulated 3PGA. In addition, artificially raising the pH in developing forespores caused a loss in the 3PGA pool, while spores from a strain overproducing phosphoglycerate mutase contained a significantly reduced level of 3PGA. These data suggest that the forespore pH decrease is responsible for 3PGA, possibly by inhibiting the pH sensitive phosphoglycerate mutase.

3. Publications

1. Illades-Aguiar, B. and P. Setlow, Studies of the processing of the protease which initiates degradation of small, acid-soluble proteins during germination of spores of Bacillus species, J. Bacteriol. 176, 2788-2795 (1994).
2. Setlow, P., Mechanisms which contribute to the long-term survival of spores of Bacillus species, J. Appl. Bacteriol. 176 (Symposium Supplement), 49S-60S (1994).
3. Fairhead, H., B. Setlow, W.M. Waites and P. Setlow, Small, acid-soluble proteins bound to DNA protect Bacillus subtilis spores from killing by freeze-drying, Appl. Env. Microbiol. 60, 2647-2649 (1994).
4. Illades-Aguiar, B. and P. Setlow, The zymogen of the protease that degrades small, acid-soluble proteins of spores of Bacillus species can rapidly autoprocess to the active enzyme in vitro, J. Bacteriol. 176, 5571-5573 (1994).
5. Illades-Aguiar, B. and P. Setlow, Autoprocessing of the protease that degrades small, acid-soluble proteins of spores of Bacillus species is triggered by low pH, dehydration and dipicolinic acid, J. Bacteriol. 176, 7032-7037 (1994).
6. Setlow, P., Mechanisms for the prevention of damage to the DNA in spores of Bacillus species, Ann. Rev. Microbiol. 49, 29-54 (1995).
7. Setlow, B. and P. Setlow, Small, acid-soluble proteins bound to DNA protect Bacillus subtilis spores from killing by dry heat, Appl. Env. Microbiol. 61, 2787-2790 (1995).
8. Popham, D.L., S. Sengupta and P. Setlow, Heat hydrogen peroxide, and UV resistance of Bacillus subtilis spores with increased core water content and with or without major DNA binding proteins, Appl. Env. Microbiol. 61, 3633-3638 (1995).
9. Sabli, M.Z.H., P. Setlow and W.M. Waites, The effect of hypochlorite on spores of Bacillus subtilis without small acid soluble proteins, Lett. Appl. Microbiol. 22, 405-407 (1996).
10. Popham, D.L. and P. Setlow, Phenotypes of Bacillus subtilis mutants lacking multiple class A high-molecular-weight penicillin-binding proteins, J. Bacteriol. 178, 2079-2085 (1996).
11. Magill, N.G., A.E. Cowan, M.A. Leyva-Vazquez, M. Brown, D.E. Koppel and P. Setlow, Analysis of the relationship between the decrease in pH and accumulation of 3-phosphoglyceric acid in developing forespores of Bacillus species, J. Bacteriol. 178, 2204-2210 (1996).
12. Setlow, B. and P. Setlow, Role of DNA repair in Bacillus subtilis spore resistance, J. Bacteriol. 178, 3486-3495 (1996).
13. Setlow, P., Endoprotease GPR, In Handbook of Proteolytic Enzymes (A.J. Barrett, N.D. Rawlings, and J.F. Woessner, Jr. (ed.)), Academic Press, London in press (1996).
14. Setlow, P. and E. Johnson, Spores and their significance, In Food Microbiology (L. Beuchat, M. Doule and T. Montville (ed.)), American Society for Microbiology, pp. 30-65 (1997).

15. Pedersen, L.B., C. Nessi and P. Setlow, Most of the propeptide is not needed for the stability and autoprocessing of the zymogen of the germination protease (GPR) of spores of Bacillus species, J. Bacteriol. in press (1997).
16. Setlow, B., C.A. Setlow and P. Setlow, Killing of bacterial spores by organic hydroperoxides, J. Indust. Microbiol. in press (1997).

4. Inventions

None.

5. Scientific Personnel Supported

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